IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
Michael EISENHUT et al.

Application No. 09/781,080
Confirmation No. 9550

Filed: February 14, 2001

For: OLIGONUCLEOTIDE CONJUGATES

Group Art Unit: 1635

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DECLARATION UNDER 37 C.F.R. 51.132

Commissioner for Patents
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Sir:

I, the undersigned co-inventor, do hereby state:

1. I am a co-inventor of claims 1 13 and 15-18 of the patent application referenced hereinabove and of the subject matter described and claimed therein.

2. We demonstrated that delivery of an oligonucleotide (antisense) conjugated to a somatostatin analog would improve the specific delivery of antisense molecules to a selected target (i.e., targets that over-express somatostatin receptors). Further, we were able to show that, through binding experiments using rat cortex membranes, that such conjugates bind to somatostatin receptors (SSTRs) with high affinity similar to that for unconjugated octreotide, a somatostatin analog, as confirmed by the IC_{50} values.

Moreover, we were able to demonstrate that delivery/uptake of conjugated antisense oligonucleotide is significantly (statistically) greater than that of a non-conjugated oligonucleotide in tumors expressing SSTRs.

3. A comparison between IC_{50} values for conjugates as described in the application versus those described for Nagy et al. is provided below. Further, a bar graph demonstrating tissue uptake of the conjugate showing unexpected accumulation of said conjugate in a tumor expressing SSTR (i.e., approximately 10-fold), which would not be expected from the data of Lu et al., is provided.

Materials and Methods

Binding assays:

For accurately determining the competitive displacement reaction, the concentration of the conjugates was determined by means of the millimolar absorption coefficient. In this regard, it was assumed that the ε_m is the ODN ε_m and the peptide ε_m: $\varepsilon_m = \Sigma$ ((nA x 15.4 + nC x 7.3 + nG x 11.7 + nT x 8.8) x 0.9) + nTrp x 5.0 + nTyr x 1.4 + nPhe x 0.2) using this equation the following ε_m values were determined: **5** = 180.5; **6** = 212.7 and **7** = 180.5.[1] For the binding assays, rat cortex membranes were resuspended at a protein concentration of 500 µg/ml in incubation buffer (10 nM HEPES, pH 7.6, with 5% BSA Fraction V, $MgCl_2$ (10 mM) and bacitracin (20 µg/ml)). 100 µg of protein were used per assay. The cell membranes (200 µl) were mixed with 30 µl of incubation buffer with increasing concentrations of the competitor (conjugates **5-7**) (10^{-5} to 10^{-10} mol/l). About 20,000 cpm ^{125}I-Tyr^3 octreotide (about 20 pm) in 70 µl incubation buffer were added. After 1 h at room temperature, the incubation was terminated by rapid filtration over "GF/B" glass fiber filter (Whatman, Springfield Mill, USA) which had been moistened with 1% BSA-containing buffer. The filters were washed with ice-cold buffer (10 mM Tris, 150 mM NaCl) and the bound radioactivity was determined by a gamma counter. The non-specific binding was determined to be about 10 – 20% of the total binding by measuring binding in the presence of excess non-labeled octreotide (10^{-6} mol/l). The specific binding was defined as

[1] See Figure 3 of the above referenced application for sequence information.

total binding minus non-specific binding. The results are shown as values of the specific binding determined from three experiments.

Tumor Analysis:

A cell suspension of the CA20948R tumor in a nutrient mixture was subcutaneously administered into the nape of the neck of male Lewis rats. After about 10 days, the tumors had grown to a volume of about 5 ml. The ^{125}Labeled compound[2] was injected into the tail vein of the animals (groups of three animals). After 1 h, the animals were sacrificed and the activity concentration of the dissected organs was determined in a gamma counter.

Results

Binding data.

TABLE 1: Comparison of binding affinities between conjugate and free carrier (i.e., the somatostatin analog, octreotide).

Conjugate of Eisenhut et al.	IC_{50}	Conjugates of Nagy et al.[3]	IC_{50}
octreotide (Control)	1.98	RC-121 (Control)	0.31
octreotate-conjugate 5	1.83 ± 0.17 nM	AN-162	2.96

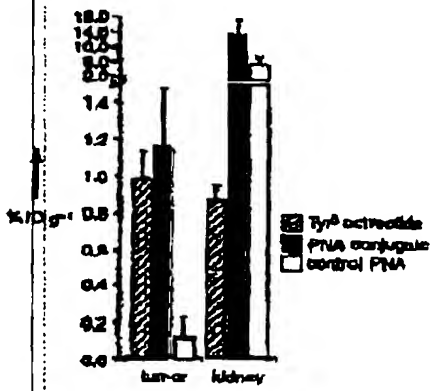
[2] (-Tyr-AGCGTGC:GCCATCCC-D-Phe-cyclo-[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-OH

[3] Nagy et al., "Synthesis and Biological Evaluation of Cytotoxic Analogs of Somatostatin Containing Doxorubicin or its Intensely Potent Derivative, 2-pyrrolinodoxorubicin." Proc. Natl. Acad. Sci. USA (1998) 95:1794-1799, Table 1, at page 1795. RC-121 = D-Phe-cyclo-[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr NH_2; RC-160 = D-Phe-cyclo-[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2. AN-162 =DOX-14-O-glt-RC121; AN-238 = 2-pyrrolino-DOX-14-O-glt-RC-121; AN-163 = DOX-14-O-glt-RC-160; AN-258 = 2-pyrrolino-DOX-14-O-glt-RC-160.

octreotate-conjugate 6	2.52 ± 0.43 nM	AN-238	23.8
octreotate-conjugate 7	1.88 ± 0.47 nM	RC-160 (Control)	1.74
-	-	AN-163	7.88
-	-	AN-258	80.1

The data from Table 1 shows that the general trend for the conjugates of Nagy et al. is reduced binding affinity, while the conjugates of the present application show no significant difference in binding affinity compared to the somatostatin analog alone.

Below is a bar graph showing data for tumor bearing Lewis rat, given in percent of the injected dose per gram of tissue (%ID g^{-1}) ± standard deviation 1 h after intravenous injection (average values from three or six animals, compounds were labeled with ^{125}I).



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The bar graph demonstrates that conjugation of the peptide moiety causes a strongly increased accumulation of the PNA oligomer in the tumor tissue (statistical significance in student's t-Test: $p = 0.021$).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issuing therefrom.

Further, Declarant sayeth not.

Sept. 22, 2003
Date